HORMONAL REGULATION OF ALTERNATIVE REPRODUCTIVE STRATEGIES

By

Tawna Morgan

RECOMMENDED:

Advisory Committee Chair

Chair, Department of Biology & Wildlife

APPROVED:

Dean, College of Natural Science and Mathematics

Dean of the Graduate School

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Tawna Morgan

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Abstract

Variation in reproductive behavior permits individuals to optimize fitness given their genetics, physiological condition, social status, age and current environmental conditions. For species with alternative reproductive strategies, behavioral differences are driven by natural and sexual selection and further mediated by the endocrine system. In this study, I explore endocrinological mechanisms associated with alternative reproductive strategies in the ruff (*Philomachus pugnax*). Ruffs breed on leks, where males use one of three genetically determined alternative reproductive strategies or phenotypes. Resident males rely on male-male aggression, colorful plumage, large body size and intensive display activity to establish courts on a lek and attract females. Satellite males operate outside the dominance hierarchy of the lek, relying on semi-cooperative displays with residents, lighter display plumage coloration and agility to access receptive females. Males using the third male strategy, considered "sneakers" or "female mimics", completely forgo typical male breeding plumage, territoriality and obvious display behaviors to obtain clandestine access to females. The differences in level of aggression and display behaviors among phenotypes suggest predictable differences in steroid hormone concentrations, which play a critical role in breeding behavior in birds. I therefore examined seasonal hormone profiles of male ruffs and applied predictions from the challenge hypothesis, which posits circulating androgen concentration will vary in relation to intensity of male-male competition during the breeding season. Consistent with the challenge hypothesis, androgen levels were higher during periods of lek instability in territorial resident ruffs. In contrast, when housed in

large flocks, androstenedione was elevated during the breeding season in satellite ruffs and followed the pattern typically observed for testosterone in polygamous birds. In response to distinctly different seasonal androgen and androstenedione profiles, I further examined the activational role of androstenedione on satellite male breeding behavior. Androstenedione stimulated both resident and satellite typical behaviors consistent with genetic morph, and thus did not specifically activate the satellite phenotype.

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General Introduction

Variation in reproductive behavior permits individuals to optimize fitness given their genetics, physiological condition, social status, age, and the current environment. Social behavior is a dynamic process that relies on genetics and the environment critically linked through physiological mechanisms. The endocrine system plays a key role in the nexus between a stimulus and expression of situation-appropriate behaviors. As messengers within the body, steroid hormones may be released into circulation rapidly, serving as a signal to specific target tissues. When target tissues are located in regions of the brain associated with breeding behaviors, steroid hormones facilitate the activation of social behaviors appropriate for successful breeding (Nelson, 2003). Genetically driven changes to hormone receptor densities, enzyme activity and capacity of hormone production can alter entire suites or individual behaviors that increase or decrease reproductive fitness (Hau, 2007). As a link between genotype and the environment, the endocrine system provides a mechanistic platform for evolution.

Reproductive behaviors vary across individuals, populations, and taxa, as well as within individuals over time. The level of variation depends on both the evolution of mating systems adapted to specific environments and the plasticity of behavior. Within the order Aves, there is considerable variation in mating systems, but the physiological mechanisms underlying mating behavior are conserved. The endocrine system plays a critical role in facilitating differences in behavior among polygamous, monogamous, cooperative, and colonial breeding systems (Hirschenhauser et al., 2003; Peters et al., 2001; Smith et al., 2005; Wingfield et al., 1991). Androgens, in particular, show distinct

patterns within mating systems (Hirschenhauser et al., 2003) and even individuals within a species (Khan et al., 2001).

Within a single species, mating behavior variation comes in two forms. A single individual may alter its breeding behavior within or among years in a manner that is referred to as tactic switching or using alternative reproductive tactics (Gross, 1996). Tactic switching is generally done in response to environmental or social conditions, and closely tied to temporal fluctuations in hormone concentration during the breeding season (Knapp et al., 2003). In contrast to the behavioral plasticity observed with tactic switching, alternative reproductive strategies refer to situations in which the ontogenetic mechanism resulting in suites of reproductive characters produce discrete alternative phenotypes, even in similar environments, resulting in fixed variation in reproductive behaviors between individuals (Gross, 1996). An individual may still be capable of tactic switching (Denardo and Sinervo, 1994), but will do so differently depending on strategy, which remains constant throughout the individual's life.

Hormones play a critical role in facilitating the evolution and expression of behavioral tactics and strategies. Steroid hormones serve as a signal connecting the perception of a stimulus to expression of situation-appropriate behaviors. They can operate during development to permanently change neural and endocrine structures such that chemical and behavioral responses to a given stimulus will differ between individuals (Astheimer et al., 1994; Jawor et al., 2006; Moore et al., 2002). Such organizational effects are a common pathway leading to development of fixed behavioral differences between sexes and may also lead to different strategies within a sex (Adkins-

Regan, 2007; Hews et al., 1994; Hews and Moore, 1996). Less static behaviors that vary within an individual are commonly influenced by temporal fluctuations in hormone concentrations (Moore, 1991). These activational effects are often temporary, such as an increase in testosterone at the beginning of the breeding season to activate breeding behavior in males and subsequently influence the intensity of territorial activity as the season progresses. Fluctuations in the same hormone may also be responsible for shifts in behavior from mate defense to parental duties (Clotfelter et al., 2007; Van Duyse et al., 2002; Van Roo, 2004). Both organizational and activational effects may be important in facilitating the evolution and expression of alternative reproductive strategies (Denardo and Sinervo, 1994; Sinervo et al., 2000).

Alternative mating strategies evolve when some individuals develop new behaviors that permit them to have higher mating success than would traditional behavior (Schuster and Wade, 2003). The close association between behavior and reproductive fitness means that during the mating season reproductive behaviors are under direct selection. Genetically influenced changes in mating behavior have an immediate effect on reproductive success and fitness. While organizational effects may play an important role in development of alternative reproductive strategies, exploring activational effects of hormones during the breeding season offers a way to address endocrinological characteristics while they are currently subject to sexual selection. Endocrinological changes during the breeding season may directly alter reproductive success. Pinpointing key endocrinological differences provides a methodology for identifying characters

currently under selection and those that evolve as correlated traits (Ketterson and Nolan, 1999).

The evolutionary constraints hypothesis posits that life history trade-offs during the breeding season are modulated by changes in circulating androgen concentrations, subsequently influencing large suites of behaviors (Hau, 2007). However, in some organisms, disassociation between circulating hormone concentrations and trait responsiveness may occur (Wiley and Goldizen, 2003), permitting individual characteristics to evolve independently of others (Hau, 2007). For species or phenotypes that maximize fitness through high annual survivorship and low annual reproductive output reducing physical costs during the breeding season may drastically reduce fitness by decreasing subsequent survivorship. Separation of trait responsiveness and circulating hormone concentration would provide a mechanism to modulate costs associated with reproduction, thus permitting resource allocation for survivorship. Dissociation of some breeding behaviors and androgen responsiveness would be one mechanism that could facilitate evolution of alternative mating behaviors.

The ruff (*Philomachus pugnax*) is a polygynous lekking sandpiper. Three genetically determined, alternative male reproductive strategies have been described (Hogan-Warburg, 1966; Jukema and Piersma, 2006; Lank et al., 1995, Lank unpublished), each with a unique set of physical and behavioral characteristics that define an alternative mating phenotype. Independent males, called resident males once they join a lek, actively defend small territories ("courts") and frequently perform mating displays using elaborate breeding plumage; satellite males do not defend territories but have

elaborate display plumage and will display on resident male territories; faeder males make up a small percentage of the population, do not defend territories or display, lack elaborate plumage, but associate with females or displaying males. The most distinct behavioral differences between strategies are thus levels of aggression and display frequencies. Only resident males establish courts by actively chasing and fighting with other resident males. Aggression frequency and intensity are highly reduced in satellite males and undetected in faeders. Behavioral differences are complemented by morphological differences among phenotypes. During the breeding season, both independent and satellite males grow elaborate ruffs and wattles of different colors and patterns that are used in mating displays and signal individual identity (Dale et al., 2001). However, faeders lack elaborate breeding plumage and are nearly identical in appearance to, and only slightly larger than females (Jukema and Piersma, 2006). Of the three phenotypes, resident males are the largest, followed by satellites then faeders (Höglund and Lundberg, 1989; Jukema and Piersma, 2006).

Although many characters define an alternative phenotype in ruffs, not all may have evolved due to the same selective pressures. Elaborate and distinct plumage may have evolved to facilitate rapid recognition within a lek (Dale et al., 2001). Larger body size in independent males may permit them to isolate and defend a female, while smaller bodied satellite and faeder males rely more on agility and mounting speed. Identifying the mechanism underlying behavioral differences among phenotypes provides insight into how sexual selection may have shaped phenotypic differences.

In this study I attempted to understand the hormonal mechanisms underlying differences in reproductive behavior in the ruff. The fixed behavioral phenotypes of ruffs are autosomally inherited (Lank et al., 1995; Lank et al., 1999). In identifying the mechanistic basis for behavioral differences among strategies, we may further identify precise characters under selection. Our approach was to first explore naturally occurring differences in hormone concentrations among behavioral phenotypes, identify specific behavior-hormone associations, and finally experimentally test the role of specific hormones on expression of suites of behaviors assigned to each behavioral phenotype. The results from this study are placed in the context of seasonal hormone dynamics found throughout various mating systems. Specifically, chapter one describes seasonal steroid hormone profiles for the two most common male behavioral strategies (independent and satellite). Chapter two focuses on hormone differences found during the breeding season among all three behavioral phenotypes and develops specific associations between behavioral characteristics and hormone concentration. Chapter three describes an experimental manipulation to further test behavior-hormone associations and identify endocrinological differences among strategies.

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Chapter 1 Seasonal Hormone Dynamics of a Lekking Shorebird

Abstract

Hormones play an important role in stimulating breeding behavior in birds. Elevated androgen concentration during the breeding season may promote breeding success through stimulation of mate attraction and defense behaviors but come at the cost of reduced parental care behaviors, immune function and higher energetic demands. Seasonal hormone profiles reflect the balance of these costs and benefits. For species with alternative reproductive strategies, seasonal hormone profiles further reflect the different mechanisms that allow individuals with distinctly different breeding strategies to balance costs associated with breeding and self maintenance. In this study, we document seasonal androgen profiles for two morphs of a high latitude, polygamous breeding shorebird with three alternative male reproductive strategies. Circulating androgen (T and DHT) concentration did not differ among strategies or exhibit a seasonal increase during the breeding season. During the breeding season, androstenedione concentration was higher in the non-territorial "satellite" morph than the territorial "resident" morph.

Keywords: ruffs, steroid hormones, androgens, testosterone, androstenedione, seasonal hormone profiles

Introduction

Activational effects of steroid hormones influence the seasonal onset of breeding behaviors in vertebrates and the development of secondary sexual characteristics (Adkins-Regan, 2005). The duration of hormone presence, as well as the plasma concentration and excretion pattern can be strongly influenced by the type of mating system (Hirschenhauser et al., 2003). However even within an individual, seasonal hormone profiles may differ among years or in response to environmental and social conditions (Vitousek et al., 2008).

In polygamous mating systems, testosterone typically increases in males at the beginning of the mating season and remains high throughout the breeding season (Goymann et al., 2007). Leks represent the extreme of the polygamous spectrum in that males defend territories but provide no external resources to mated females and must continuously compete for mates, with little time spent seeking out resources even to maintain themselves (Bachman and Widemo, 1999). Given the intense and continued male-male competition, the seasonal androgen profile of males in a lek breeding system is expected to increase at the onset of lek formation and remain high until disintegration of the lek.

Ruffs are polygamous lek breeding shorebirds, with genetically determined variation in the use of three mate acquisition strategies. Resident male ruffs defend small courts on a lek, attracting females through high display frequency and agonistic interactions with competing resident males. Satellite ruffs also display on leks to a lesser

degree but do not defend a court. Their mating success is largely dependent on forming temporary associations with resident males (Hugie and Lank, 1997; Widemo, 1998) and speed in mounting soliciting females. Although both behavioral phenotypes operate through a polygamous mating system, the distinct lack of aggression and territorial defense in satellite males is atypical of polygamous breeding systems.

In this study we seek to describe the seasonal androgen profile of a lekking shorebird with alternative reproductive strategies. We focus on total androgen, which includes dihydrotestosterone (DHT) and testosterone (T). Seasonal testosterone profiles of other polygamous species provide a reference point for comparison with patterns in resident and satellite ruffs. We also investigate circulating levels of androstenedione, a precursor to T and DHT, which is less well understood, but may provide a second mechanism facilitating mating behavior when aggression and territorial defense play a smaller role in mate acquisition.

Methods

In 2003, we collected blood plasma samples from 20 male ruffs housed in an outdoor aviary at Simon Fraser University, Burnaby, British Columbia. Males were housed in groups of 10 including 5 residents and 5 satellites. Group composition was consistent over the breeding season, although the groups were large enough to permit several leks to form, whose participants changed throughout the breeding season. Females were housed separately from males, but were visually accessible.

Starting 14 March, well before the commencement of the breeding season, we collected blood samples from all resident and satellite males every two weeks, between

0900 and 1400, until 9 July (see Figure 1.1 for collection dates). Approximately 400 µl of blood were collected from the brachial vein using a 26 g needle attached to a 1 ml syringe. Samples were centrifuged at 5000 rpm for 6 minutes, and plasma was collected and placed in a -20◦ C freezer until hormone assays were performed. Hormone analysis was restricted to 10 males between 3 and 9 years of age who actively exhibited breeding behavior and nuptial plumage during the breeding season. The potential breeding status of each male, as judged by the development or loss of his display feathers and seasonally developed facial wattles, was also recorded when bled, and serves as a record of the number of actively breeding males present.

Plasma samples were analyzed in 2007 at the University of Alaska, Fairbanks. All samples were assayed in duplicate following the procedures of Goymann and Wingfield (2004) and Wingfield and Farner (1975). A subset of 30 samples, collected during the breeding season (April 28 thru June 9), were extracted with dichloromethane and separated on diatomaceous earth/glycol columns, such that dihydrotestosterone (DHT), testosterone (T) and androstenedione (AE) could be collected from a single sample. Given the strong correlation between T and DHT and complications associated with extracting multiple hormones from a single sample, remaining samples (n = 77), collected pre and post breeding, were assayed for total androgen concentration using a testosterone antibody that binds to both T and DHT (Sigma) and a second antibody that binds only to AE (Sigma). Percent recovery for androgens ranged from 59 – 88% and AE ranged from 52-89%. Minimum detectability was 0.29 ng/ml for androgens and 0.59 ng/ml for AE. Inter assay coefficient of variation was less than 25% for AE and

androgens. DHT was analyzed in a single assay. Intra assay coefficient of variation was less than 10% for all assays.

To determine the influence of strategy on hormone concentration, we used a repeated measures mixed effect model in Proc Mixed, SAS® 8.1 (SAS Institute 2006) with individual as a random factor. Posthoc comparisons were used to identify differences between strategies on each sampling date, independent of all other observations. Date and strategy were treated as categorical variables. A second group of models was run for both AE and androgens in Proc Mixed, SAS® 8.1 (SAS Institute 2006) in order to detect seasonal patterns. Date was transformed into ordinal day of the year for these models. Models included day, the categorical variable of strategy and the interaction of these terms. Hormone concentrations were log transformed to meet assumptions of normality. Generalized logit models (SAS Proc CATMOD) were used to assess the influence of age on day of peak hormone concentrations. Age and day of peak hormone concentration were treated as continuous variables.

Results

A subset of plasma samples (n=30) from 10 actively breeding males were collected from 28 April and 9 June 2003 and were analyzed for T, DHT and AE concentration. Neither date ($F_{4,13} = 0.38$, $P = 0.82$ for T; $F_{4,13} = 0.65$, $P = 0.64$ for DHT), nor strategy ($F_{1,8} = 0.87$, $P = 0.37$ for T; $F_{1,8} = 0.48$, $P = 0.51$ for DHT) nor the interaction of date and strategy ($F_{3,13} = 1.14$, $P = 0.37$ for T; $F_{3,13} = 1.33$, $P = 0.31$ for DHT) were significant predictors of DHT or T concentration (Table 1.1, Figure 1.1a).

DHT and T concentrations were significantly correlated (R^2 =0.75, $P < 0.01$), and not significantly different from each other ($t = 0.38$, $df = 58$, $P = 0.71$).

AE varied among dates ($F_{4,13} = 3.60$, $P = 0.03$) and between strategies ($F_{1,8} = 19.39$, $P < 0.01$), but the date by strategy interaction was not significant ($F_{2,13} = 2.8$, $P = 0.08$) (Table 1.1, Figure 1.1b). On 28 April, 12 May, and 26 May satellite males had higher AE concentrations than residents ($F_{1,13} = 11.90$, $P < 0.01$; $F_{1,13} = 6.49$, $P < 0.02$; $F_{1,13} = 13.48$, $P < 0.01$).

For models used to detect seasonal trends, neither day nor strategy was significant in predicting androgen concentration during the breeding season (Table 1.2). However, for AE, strategy ($F_{1,8} = 5.69$, $P = 0.04$) was a significant predictor of AE profiles during the breeding season (Table 1.2).

Pre- and post-breeding plasma samples were collected from the same males on 14 and 28 of March (pre-breeding), and 23 June and 9 July (post-breeding). These samples were assayed for total plasma androgens, which included T and DHT combined, and AE. Because pre- and post-breeding samples were analyzed using different laboratory procedures than those for breeding season samples, they were considered separately. There was no significant difference in androgen or AE concentration between resident and satellite males during the pre- and post-breeding season (Table 1.1, Figure 1.1).

Both resident and satellite males showed individual variability in the timing of peak androgen and AE concentration (Figure 1.2). The greatest number of satellite males

Table 1.1 Results for mixed effects model of seasonal differences in androgen and androstenedione concentration in resident and satellite male ruffs.

Seasonal Differences

Breeding	Numerator df	Denominator df	F	P
Testosterone (n = 30)				
Strategy	1	8	0.87	0.28
Date	4	13	0.38	0.82
Strategy * Date	3	13	1.14	0.36
Androstenedione (n = 30)				
Strategy	1	8	19.39	<0.01
Date	4	13	3.60	0.03
Strategy * Date	3	13	2.80	0.08
Pre and Post Breeding				
Androgens (n = 73)				
Strategy	1	8	0.74	0.42
Date	1	28	0.35	0.56
Strategy * Date	1	28	0.73	0.40
Androstenedione (n = 73)				
Strategy	1	8	0.00	0.97
Date	1	29	1.04	0.32
Strategy * Date	1	29	0.00	0.96

Table 1.2 Results for mixed effects model of seasonal androgen and androstenedione dynamics for resident and satellite male ruffs.

Seasonal Dynamics

	Numerator df	Denominator df	F	P
Testosterone (n = 30)				
Strategy	1	8	0.28	0.61
Date	1	18	0.00	0.96
Strategy * Date	1	18	0.14	0.71
Androstenedione (n = 30)				
Strategy	1	8	5.69	0.04
Date	1	18	1.53	0.23
Strategy * Date	1	18	3.16	0.09

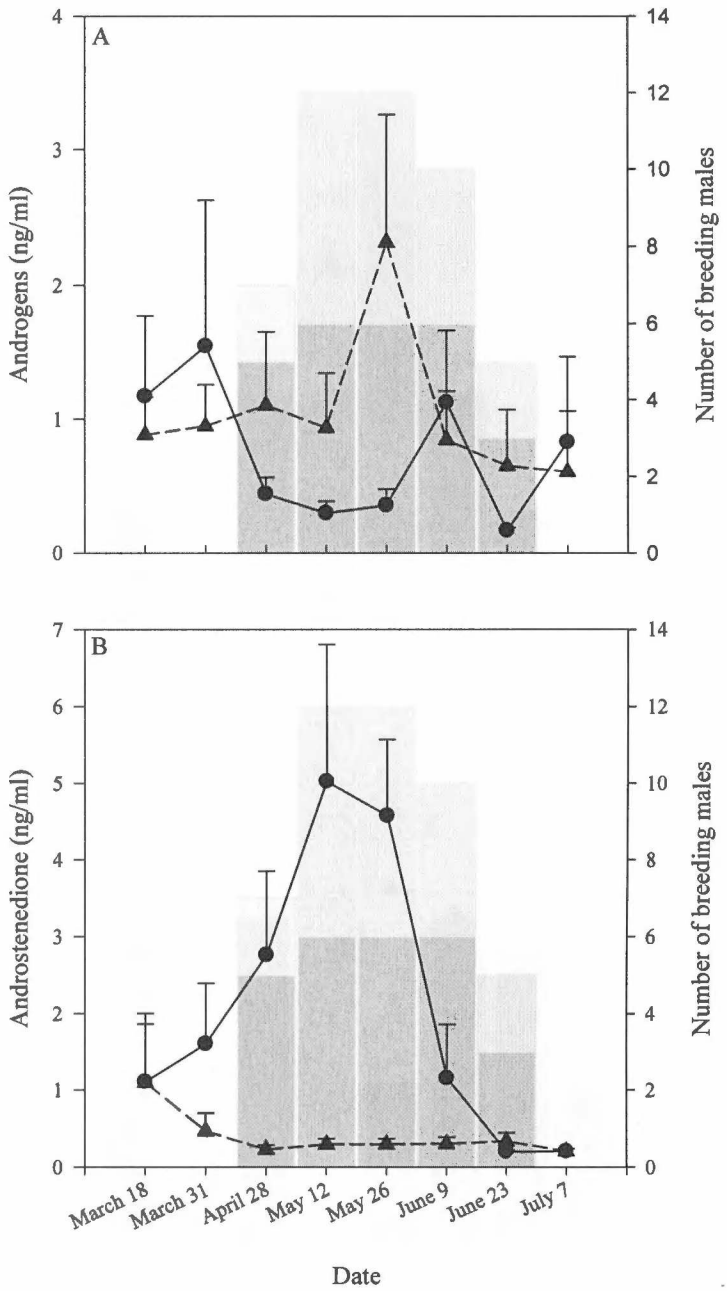

Figure 1.1 Mean plasma gonadal androgens (A) and androstenedione (B) concentrations for resident (triangles) and satellite (circles) male ruffs in 2003 (n = 10). Stacked bars indicate the number of males in full breeding plumage on each sampling date, residents (dark grey) and satellites (light grey). Error bars indicate standard error.

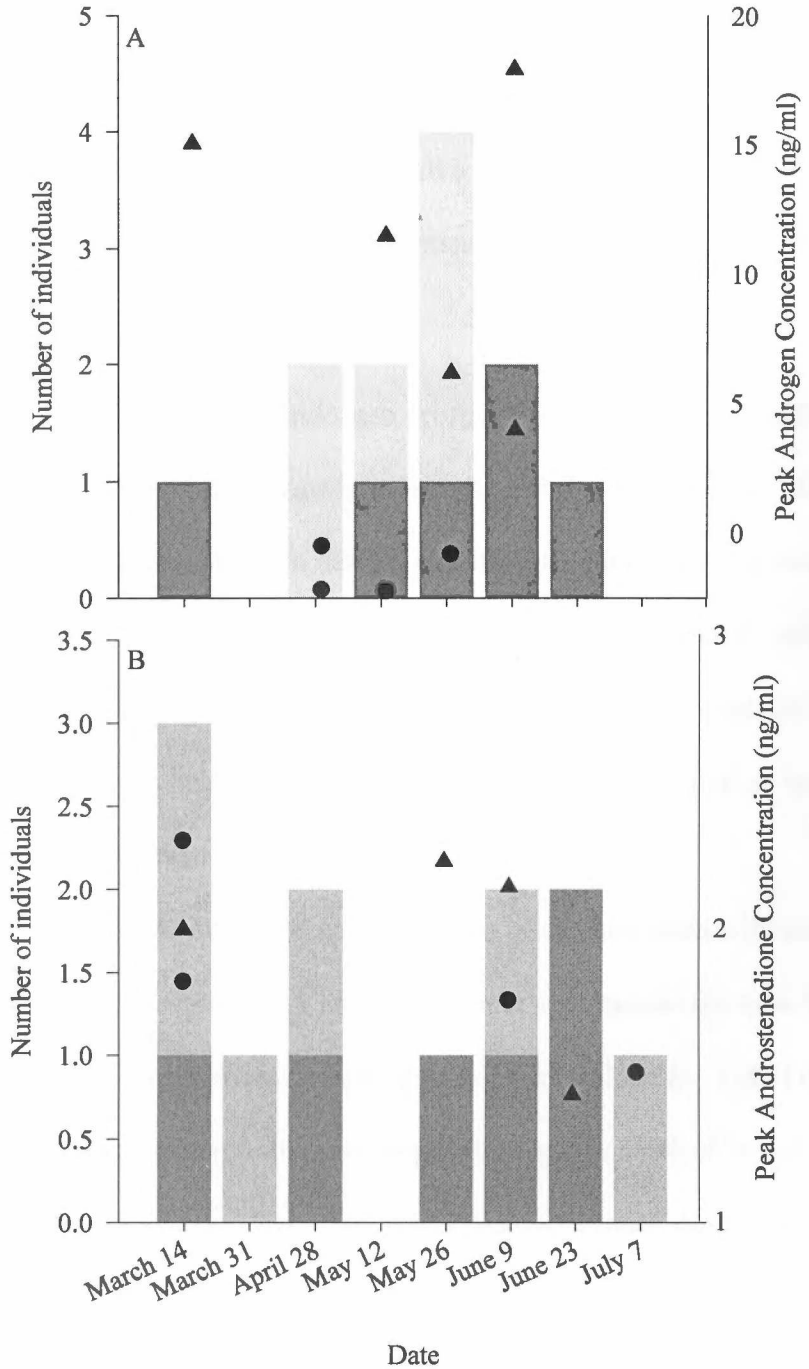

Figure 1.2 Timing of peak gonadal androgens (A) and androstenedione (B) concentration for resident (triangles and dark grey bars) and satellite (circles and light grey bars) males during 2003.

had peak androgen concentrations when the number of breeding males was highest. Resident males had higher variability in timing of peak androgens, with a large number having peak concentrations during the pre-breeding season and approaching the end of the breeding season. Age was not a significant predictor of timing of peak androgen (F = 2.51, df = 1, P = 0.16,) or androstenedione (F = 4.43, df = 1, P = 0.07) concentration.

Discussion

The seasonal androgen profiles found in this study differ from those typically observed in other polygamous breeding birds (Steiger et al., 2006). Most profoundly, androgen concentration did not significantly increase at the onset of the breeding season, nor was it elevated throughout the entire period of territory defense. In contrast androstenedione more closely conformed to androgen profiles observed in other polygamous breeding birds. AE concentration had a distinct seasonal pattern, with higher concentrations in satellite than resident males.

While increased androgens are often associated with seasonal breeding in birds, there are exceptions. A lack of seasonal testosterone increase has been observed in monogamous colonial breeding gulls (Wingfield et al., 1982) in which aggression does not play a large role in mate acquisition, and in tropical breeding birds (Moore et al., 2004). In addition, testosterone concentration may show high individual variation, particularly in lek breeding systems (Alatalo et al., 1996), in which a single male may acquire most of the copulations during a given time period (Vitousek et al., 2008). The intensity of male-male competition within leks may preclude an individual's ability to continue displaying throughout the breeding period (Bachman and Widemo, 1999).

Elevated testosterone concentration is most beneficial when direct competition is necessary. The relatively stable hierarchy of a ruff lek may reduce the need for enhanced androgen secretion to times when the hierarchy is challenged and a male must defend his position. In this manner the period of elevated androgen concentration would be reduced and vary between individuals. The high variability in timing of peak androgen concentrations among males could preclude the overall seasonal profile within the population. Further examination of breeding behavior and androgen concentration within an individual would provide insight into the seasonal dynamics of androgens in the ruff.

Aggression is not a typical characteristic associated with mate acquisition in satellite male ruffs; thus we did not expect gonadal androgen profiles to match those of other polygamous mating species. Despite less individual variability in the timing of peak androgen concentrations in satellite males, androgen concentration did not show a clear seasonal increase in association with breeding activities. While individual variability in resident male ruffs may preclude a clear seasonal increase in circulating androgen concentration, a lack of seasonal increase in satellite males may be due to differences in the endocrinological pathway. Satellite males may lack the stimulus to increase circulating androgen concentration or have a reduced capacity to produce and secrete androgens (Moore et al., 2002).

The distinct difference between resident and satellite male ruffs in androstenedione profiles has not been reported in other species with alternative reproductive strategies. As a precursor molecule in the synthesis of both estrogens and

androgens, androstenedione's role in stimulating reproductive behaviors is not clear. With no known receptors of its own, androstenedione must be converted to either estrogen or an androgen to activate behavior (Nelson, 2003). However, which behaviors are stimulated is entirely dependent on the enzymes present to convert androstenedione and androgen or estrogen receptor density at target tissues. Previous work has suggested androstenedione may play a role in mate guarding (Goymann et al., 2003) in social breeding mammals, possibly via conversion to testosterone. Satellite male ruffs, do not participate in mate guarding; however, they do develop dynamic associations with resident male ruffs. Androstenedione may facilitate male-male competition for resident-satellite associations, while foregoing costs associated with elevated circulating testosterone.

Androstenedione may also suppress testosterone production. In a similar system with alternative reproductive strategies – tree lizards – the ability to tactic switch between territorial and non-territorial behaviors within a typically nomadic morph is a result of variation in sensitivity to corticosterone between territorial and nomadic morphs. In nomadic males, elevated corticosterone suppresses testosterone production, thus reducing the stimulus for aggressive and territorial behaviors (Knapp et al., 2003). Androstenedione may serve a similar role in satellite male ruffs, suppressing testosterone production and subsequent aggression.

Although the association between androstenedione and reproductive behaviors has not been described for most species, it may be an important hormone precursor modulating reproductive behavior, particularly when mate acquisition does not involve

intense male-male aggression. Elevated androstenedione concentration in satellite ruffs further highlights differences in the endocrinological pathway between these two reproductive strategies. Future research should focus on indentifying behaviors directly associated with androstenedione, differences in the chemical pathway of hormone production and behavioral sensitivity between these two phenotypes.

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Chapter 2 The Challenge Hypothesis Applied to Alternative Reproductive Strategies[1]

Abstract

The challenge hypothesis predicts that males should respond to social challenges during the breeding season with an increase in androgen concentration. For lekking species, the establishment of a social hierarchy may decrease the need for prolonged exposure to high concentrations of testosterone, limiting upregulation of the hypothalamo-pituitary-gonadal axis to periods of social instability. We tested the challenge hypothesis in a lekking shorebird with three alternative male reproductive strategies, and compared seasonal circulating steroid hormone profiles across strategies. Androgen concentration was expected to be positively related to social instability within the lek and to vary in magnitude among reproductive strategies in parallel with their reliance on agonistic interactions. Territorial "resident" males experienced higher aggression and greater social instability during the breeding season and had the highest androgen concentrations. Androgen concentration was lower in non-territorial "satellite" males which do not engage in agonistic behavior, but androstenedione was elevated. Female-mimic "faeder" males also had low androgen and the highest androstenedione concentrations. Our results support the predictions of the challenge hypothesis in a lekking shorebird. Furthermore, we demonstrate that circulating androgen concentrations

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may differ among mating strategies in the ruff, and suggest that other steroid hormones or their precursors may be equally important in modulating reproductive behavior.

Keywords: testosterone, androstenedione, corticosterone, ruff, *Philomachus pugnax*, challenge hypothesis, social behavior, alternative reproductive strategies, androgens.

Introduction

Steroid hormones play a critical role in facilitating social behaviors including breeding behaviors (Adkins-Regan, 2005). Their role in facilitating breeding behaviors has led to the development of well described relationships between steroid hormones and breeding behavior across vertebrate mating systems (Hirschenhauser et al., 2005). In this study, we test hormone-behavior relationships within a single species with alternative male reproductive strategies.

The challenge hypothesis posits that androgen concentration should vary in response to social challenges during the breeding season (Wingfield et al., 1990). Males in polygamous breeding systems with no paternal care are expected to operate near maximal testosterone capacity for most of the breeding season in response to the need for continual mate attraction and copulation opportunities. In contrast, in monogamous species, mate defense and attraction, as well as social challenges, are more limited to initial periods of territorial establishment and/or mate acquisition, and testosterone is only expected to approach maximum levels early in the breeding season (Wingfield et al., 1990; Wingfield et al., 2001). Species that do not seasonally compete for mates or breeding territories often have no increase in testosterone above baseline levels (Goymann et al., 2004; Moore et al., 2004). In this study, we test the challenge hypothesis in a polygynous lek breeding shorebird with no paternal care of young, but limited periods of social challenge. We compare androgen profiles among three male reproductive strategies that differ in their level of territorial aggression and breeding display intensity.

Male ruffs (*Philomachus pugnax*), like most lekking species, exhibit no paternal care and are highly polygynous. Uniquely, however, individual breeding males show one of three behavioral phenotypes at leks: resident, satellite or "faeder" (Hogan-Warburg, 1966; Jukema and Piersma, 2006). The phenotypes are different genetic morphs, and differ in breeding-season plumage ornamentation, body size, territorial aggression, and display activity (Lank, unpublished; Lank et al., 1999; Lank et al., 1995). Dark-plumaged "independent" males, called "residents" when they establish and defend small courts on a lek, frequently fight with other residents and perform silent display postures to attract females. In contrast, light-plumaged satellite males do not defend courts and instead develop dynamic associations with residents and display alongside them on co-occupied courts (Hogan-Warburg, 1966; Hugie and Lank, 1997; Widemo, 1997). A third morph, termed "faeders", have body sizes intermediate between those of females and ornamented males, completely lack male display plumage, do not show territorial or courtship behavior, and have disproportionately large testes (Jukema and Piersma 2006). They are thus similar in appearance to females and have been labeled female mimics. Faeders spend most of their time with female flocks or on the periphery of male courts. The behavioral variation among reproductive phenotypes led us to predict differences in seasonal hormone profiles and androgen responsiveness to social instability.

Resident males are both the targets and instigators of aggressive encounters, and their access to receptive females depends on their ability to defend a court and attract females. Within breeding leks, which typically range in size from 2-10 resident males (Höglund et al., 1993), residents establish a hierarchy, with more dominant males having

greater mating success (Widemo 1997). The majority of independent males, termed "marginal" (Hogan-Warburg, 1966) are unable to establish a court, and move among leks attempting to become residents as the opportunity arises. Throughout the breeding season, social hierarchies fluctuate between periods of stability and instability as additional males enter or leave the lek (Lank and Smith, 1987). Given the relative social stability of a lek, under the challenge hypothesis we expected resident males to have low androgen concentration throughout much of the breeding season, but respond to social challenges during periods of instability with short-term increases in androgen concentration.

In contrast, satellite and faeder males operate outside of the dominance-driven hierarchy of lekking residents. They do not threaten other males' territories and are not usually challenged by residents. Their access to females largely depends on associations with dominant resident males, their speed in mounting soliciting females, and possibly female choice, but not on male-male aggressive behaviors. Given the lack of aggression by both satellites and faeders, and the consequent stability of their social positions, androgen concentration was not expected to fluctuate in conjunction with social stability in satellite and faeder ruffs.

We evaluate androgen concentration, along with corticosterone and the steroid hormone precursor androstenedione, which may vary seasonally and in response to social conditions. Although testosterone is the most widely studied androgen associated with breeding behavior in birds, androstenedione (AE) is a androgen precursor that is secreted from the gonads or the adrenals, and may be subsequently converted to testosterone at

target tissues (Chapter 1; Matsunaga et al., 2002; Soma et al., 2003; Soma and Wingfield, 2001). In ruffs, AE may play a greater role in stimulating breeding behaviors in satellite or faeder males which lack the stimulus of territorial encounters or capacity for androgen secretion. We evaluated seasonal AE profiles for all three behavioral phenotypes in order to examine their possible roles in stimulating breeding behavior in alternative mating strategies. We also examined corticosterone (B) concentration; like testosterone, B is known to fluctuate in response to social conditions (Goymann and Wingfield, 2004a) and may inhibit behaviors typically associated with testosterone (Denardo and Sinervo, 1994). Corticosterone, therefore, may be one mechanism modulating circulating testosterone concentration and associated aggressive behaviors in satellite and faeder ruffs.

Methods

In 2006, we measured plasma hormone concentrations during the breeding season for a long-term captive population of ruffs housed in an outdoor aviary. Ten male ruffs were housed in two pens with 3 residents and 2 satellites each. Social groups remained constant throughout the breeding season. Males were permitted constant visual access to females, and physical access was provided for 2 – 3 hours between 0600 and 1100 each day, when lek attendance is highest in the wild (Lank and Smith 1987). All copulation attempts were recorded. In addition, two 15 minute behavioral observations were conducted for each pen each day. Behavioral observations consisted of scan sampling every 90 seconds to record display postures of all individuals, associations between resident and satellite males, and the presence of females within 0.5 m of a displaying

male. During the interval between scans, all fights, agonistic interactions, chases and copulations were recorded. Sample collection commenced the second week of May and continued through the first week of July. Blood sampling occurred biweekly between 1000 and 1200, immediately after males had access to females. Approximately 400 μl of blood was collected from the brachial vein using a 26 g syringe. Samples were centrifuged at 5000 rpm for 6 minutes, and plasma was collected and placed in a -20° C freezer until hormone assays were performed.

Plasma samples collected during the breeding season may reflect baseline seasonal corticosterone profiles and stress at the time of sampling; however, testing the magnitude of stress response post-breeding may reflect prolonged stress experienced over the duration of the breeding season. Baseline plasma samples collected biweekly were used to reflect a pattern of stress throughout the breeding season and detect timing of stressful events. Post-breeding season plasma samples were collected in August to determine the magnitude of the stress response, which may reflect overall conditions experienced during the breeding season. An initial sample was collected within three minutes of capture while a second sample was collected thirty minutes after capture. Between samples, individuals were held in cloth bags, in isolation from other birds or human activity.

We also collected plasma samples from two faeder males, who were housed with a flock of females and a rotating sequence of resident and satellite males. Faeder males had constant visual access to lekking males and were introduced daily to lekking males for 20-30 minutes. This was part of a concurrent experiment, but permitted us to collect

blood samples from faeders over the entire season and thus provide a seasonal profile for three steroid hormones often associated with breeding. Because we were limited to two faeder males and these males were part of a separate experiment, hormone concentrations for these individuals are not included in statistical analyses.

The challenge hypothesis predicts that unchallenged individuals should have low testosterone concentrations in relation to breeding behaviors. To explore relationships between individual hormone concentrations and social stability, we used behavioral data to calculate a social stability index based on the number of challenged versus unchallenged interactions an individual was involved in. Social stability for residents and satellites was calculated as the proportion of all aggressive behaviors (chasing, fighting, aggressive interactions, and being chased) that were unchallenged dominance events minus the proportion that were unchallenged submissive events on a given day (Lank et al. 1999). Thus, submissive individuals had a score approaching negative one and dominant individuals had a score approaching one. This score was then squared so that both completely submissive individuals and completely dominant individuals had a score of 1, while individuals involved in similar numbers of dominant and submissive events had scores approaching 0. Thus a given stability index score reflected an individual's stability in a given status rather than current social status and a score approaching 1 represented an individual who had an unchallenged position as part of a stable hierarchy.

Plasma samples were analyzed in 2007 at the University of Alaska, Fairbanks. All samples were assayed in duplicate following the procedures of Goymann and Wingfield (2004b) and Wingfield and Farner (1975). One hundred microliters of plasma

from each sample were simultaneously assayed for androgens and AE by extraction with dichloromethane and separation on diatomaceous earth/glycol columns. A 20 µl subsample was extracted with dichloromethane and assayed solely for B. Thus, multiple hormones were collected from a single sample. Samples were arranged randomly throughout assays.

Minimum detectability for hormone concentrations were 0.14 ng/ml, 0.17 ng/ml, 0.89 ng/ml for androgens, AE and B respectively. Intra assay coefficient of variation was less than 10% for all hormones. Inter-assay coefficient of variation was 21% for androgens and 22% for AE. A single assay was used to analyze plasma samples for B, so only intra-assay coefficients of variation are relevant.

To determine the influence of seasonality, behavioral phenotype, social stability and female presence on hormone concentration, we used Proc Mixed in SAS® 8.1 (SAS Institute 2006) with individual male as a random factor, and posthoc comparisons of differences between strategies on each sampling date, independent of all other observations. Date and strategy were included in all models as categorical variables, while social stability index and the number of females present were treated as continuous variables. A second group of models was run for both AE and androgens in Proc Mixed, SAS® 8.1 (SAS Institute 2006) in order to detect seasonal patterns. Date was transformed into ordinal day of the year for these models and treated as a continuous variable.

Results

Forty-four plasma samples collected between 12 May and 7 July 2006 from 10 males were analyzed for androgen, AE and B. Androgen concentration was negatively

associated with social stability ($F_{1,24} = 7.8$, $P = 0.01$; Table 2.1, Figure 2.1c and 2.2), and positively related to the number of nearby females ($F_{1,24} = 12.08$, $P < 0.01$; Table 2.1). The interaction between date and strategy was also significant ($F_{4,24} = 4.06$, $P = 0.01$; Table 2.1), with androgen concentration being higher in residents on 26 May ($F_{1,24} = 5.66$, $P = 0.03$), but not significantly different on other dates. Neither the main effects of date nor strategy were significant (Table 2.1).

For seasonal androgen profiles, day ($F_{1,32} = 17.44$, $P < 0.01$) and the interaction of day and strategy ($F_{1,32} = 10.51$, $P < 0.01$) were significant but not strategy ($F_{1,8} = 13.73$, $P < 0.06$; Table 2.2). For seasonal AE profiles, only the effect of day was significant ($F_{1,31} = 13.63$, $P < 0.01$). None of the variables included in models to predict AE concentration were significant.

None of the variables included in current models were significant in explaining B concentrations (Table 2.1 and 2.2) in resident and satellite males (Figure 2.1b). During post-breeding season sampling, both satellite and residents showed an increase in corticosterone between initial and 30-minute samples ($t = -4.29$, df = 10, $P = 0.002$). However, there was no difference in baseline ($t = 0.93$, df = 10, $P = 0.373$) or stress

Table 2.1 Results for mixed effects model of seasonal differences in androgen, androstenedione and corticosterone concentration in resident and satellite male ruffs.

Seasonal Differences

Breeding	Numerator df	Denominator df	F	P
Androgens (n = 44)				
Strategy	1	8	1.67	0.23
Date	4	24	0.66	0.63
Strategy * Date	4	24	4.06	0.01
Social stability	1	24	7.80	0.01
Number of females	1	24	12.08	<0.01
Androstenedione (n = 43)				
Strategy	1	8	1.08	0.32
Date	4	23	1.00	0.43
Strategy * Date	4	23	0.75	0.56
Social stability	1	23	0.35	0.55
Number of females	1	23	3.75	0.07
Corticosterone (n = 47)				
Strategy	1	8	0.08	0.79
Date	4	27	1.11	0.37
Strategy * Date	4	27	1.37	0.27
Social stability	1	27	0.34	0.56
Number of females	1	27	0.65	0.43

Table 2.2 Results for mixed effects model of seasonal androgen, androstenedione and corticosterone dynamics for resident and satellite male ruffs.

Seasonal Dynamics

	Numerator df	Denominator df	F	P
Androgens (n = 44)				
Strategy	1	8	13.73	0.06
Day	1	32	17.44	<0.01
Strategy * Day	1	32	10.51	0.00
Androstenedione (n = 43)				
Strategy	1	8	1.62	0.24
Day	1	31	13.63	<0.01
Strategy * Day	1	31	1.27	0.27
Corticosterone (n = 47)				
Strategy	1	8	0.11	0.75
Day	1	35	2.07	0.16
Strategy * Day	1	35	0.10	0.76

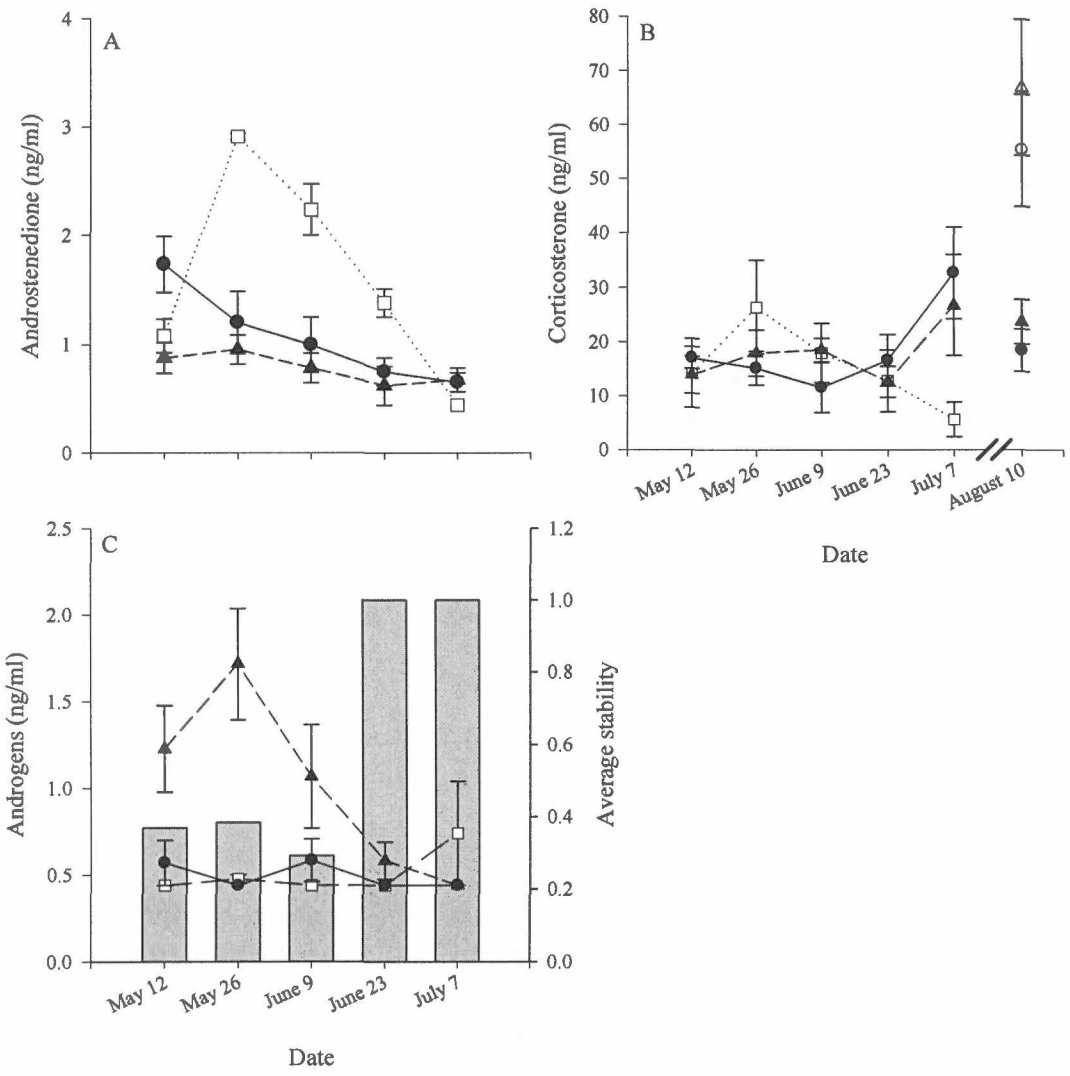

Figure 2.1 Mean hormone concentrations, with standard error, for resident (filled triangles; n=6), satellite (filled circles; n=4) and faeder (open squares; n=2) males in 2006. Open circles and triangles in graph B indicate stress induced corticosterone concentrations for satellite and resident males respectively. Gray bars in graph C represent the mean stability index for resident males. All satellite males had a stability index of 1 on all sample dates, thus are not graphed.

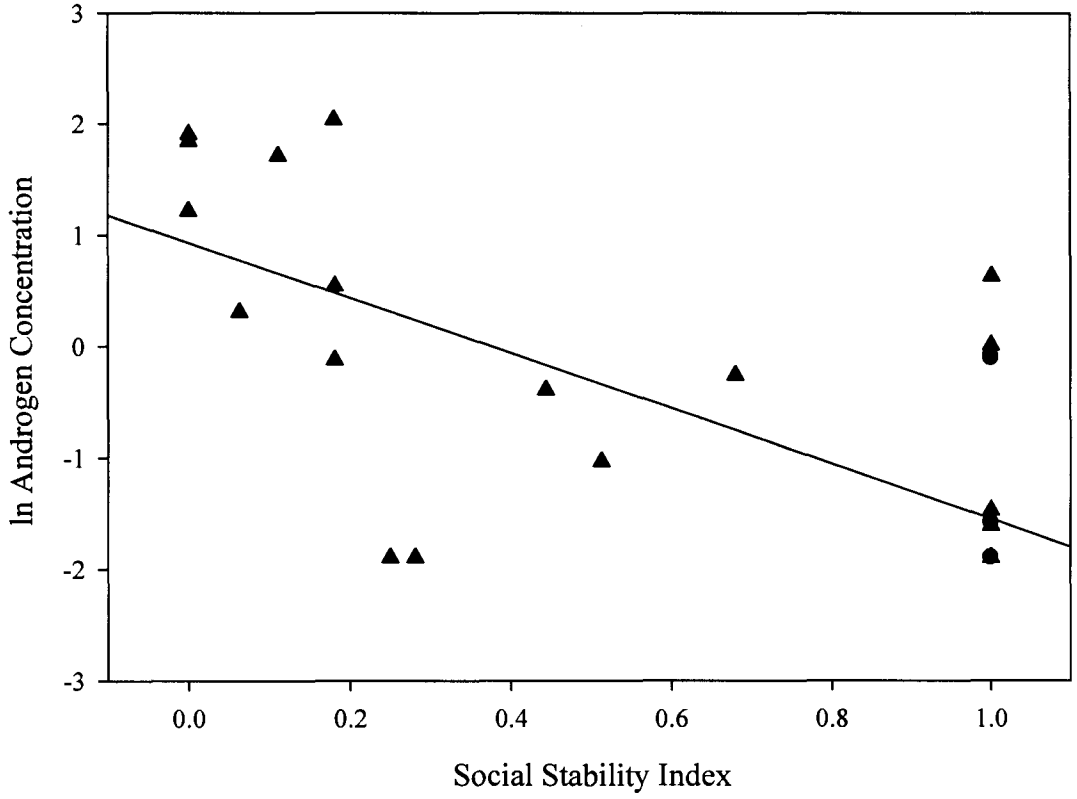

Figure 2.2 Androgen concentration vs. social stability for male ruffs. Residents are indicated with triangles and satellites with circles.

induced ($t = 0.64$, df = 9, $P = 0.536$) corticosterone concentration between resident and satellite males. Although they were not included in statistical models, the two faeder males had the highest AE concentrations recorded, low androgen, and similar B concentrations relative to the other two morphs (Figure 2.1). Neither date, strategy, nor the interaction of date and strategy was significant in explaining AE concentrations (Table 2.1).

Discussion

Our primary results are consistent with predictions from the challenge hypothesis as applied to this unusual male mating system. Androgen levels were positively related to social instability and the presence of females, but not influenced by strategy or date. Neither androstenedione nor corticosterone showed strong seasonal patterns, nor were they significantly related to social instability, the presence of females or strategy.

Leks permit the establishment of male-male hierarchies, and as such allow periods of stability to be punctuated with periods of instability, such as when a new male joins the lek, the number of individuals competing for courts increases, or when female visitation rates change. Given the costs associated with prolonged elevation of testosterone (Wingfield et al., 2001), it may be beneficial to limit androgen increases to those periods in which aggression and court defense are most profitable. The seasonal patterns in androgen concentrations support this hypothesis; however, it is difficult to distinguish seasonal changes in testosterone from androgen responses to direct male-male interaction (Goymann et al., 2007). Highest androgen concentrations occurred at the onset of lek establishment, corresponding to periods of highest social instability within

the lek. In the wild, however, period of instability may be more frequent as new individuals join leks (Lank and Smith 1987), and increases in androgen concentration may be limited not only to the onset of lek establishment.

Satellite ruffs showed lower and relatively stable concentrations of androgens throughout the season. It is unclear whether this difference is due to a lack of behavioral stimulus for satellite males, who had a stability index at or close to 1.0 for the entire season, or if satellite males lack the capacity to secrete androgens in high quantities. Satellite males operate outside of the aggression driven hierarchy of the lek; thus satellite specific behaviors associated with male-male competition may not have been detected in this study. A more intensive study of male-male competition, among satellites, for development of resident-satellite associations may further highlight hormone behavior relationships for this morph.

If androstenedione in satellites serves to facilitate traits and behaviors generally associated with androgens in territorial males, then we would expect AE to be highest when competition for mates and potential development of male-male associations was greatest. This should correspond to the onset of the breeding season through the period of highest copulation intensity. We found this pattern in seasonal AE profiles when males were housed in large flocks (Chapter 1), but not in the current study which utilized smaller flocks. Differences in AE profiles between experiments may reflect differences in experimental design and social conditions. In 2003, individuals were housed in large flocks, in which social dynamics may have been more fluid than when males were housed in small groups which formed relatively stable leks. If androstenedione mediates

competition for resident-satellite associations, the fluid social dynamics of studies conducted in 2003 may have demanded an increase in androstenedione concentration to adapt to changing social conditions. Testing the precise role of androstenedione in satellite male ruffs would require more direct experimentation.

The androgen profiles of our two faeders were more extreme versions of satellite profiles. Like the satellites, they showed stable low androgen levels and elevated and variable AE.

Despite differences in AE and androgens, we did not detect a difference in seasonal corticosterone (B) profiles. Previous studies have identified B as a potential suppresser of testosterone and territorial behavior in species with alternative reproductive tactics consisting of territorial and non-territorial phenotypes (Knapp et al., 2003). However, this difference was associated with tactic switching within the typically non-territorial phenotype. Corticosterone may provide a mechanism facilitating behavioral plasticity within strategies, but does not show consistent differences among strategies of ruffs. In addition, post breeding season sampling detected no difference in adrenal responsiveness among strategies, suggesting resident and satellite phenotypes did not vary in the extent of prolonged exposure to B during the breeding season. Elevated corticosterone concentration during the breeding season may reflect a fitness disadvantage between phenotypes (Moore et al., 1998); however we did not detect a corticosterone mediated difference in physiological cost between phenotypes in the ruff.

The observed differences in testosterone concentrations between mating strategies in ruffs support the basic premises of the challenge hypothesis, but highlight the

complexity of the interaction between androgens and competition for mates during the breeding season. As predicted, agonistic behavior and mate competition in resident males corresponded with elevated testosterone concentration; while the less agonistic satellite males did not show this relationship. Furthermore, testosterone varied as predicted with periods of social instability, and did not show the typical pattern of continuously elevated levels observed in many polygynous species (Goymann et al., 2007). Seasonal profiles in AE are not available from other studies comparison, but androstenedione concentration in satellite and faeder ruffs is higher than concentrations reported in male sex role reversed African Black Coucals (*Centropus grillii*) during the breeding season (Goymann and Wingfield, 2004). The elevated AE concentration detected in male ruffs and its apparent relationship with breeding behaviors highlights the need to further explore the role of this androgen precursor in mating behavior.

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Chapter 3 Androstenedione and the Expression of Alternative Reproductive Strategies[2]

Abstract

Hormones play a critical role in the expression of reproductive behavior. When more than one breeding phenotype has evolved within a species, the endocrine system offers a mechanism to facilitate behavioral differences. Differences in hormone levels may influence entire suites of characters, while alterations further downstream may isolate changes to individual traits. In ruffs, satellite males differ from resident males in level of aggression, display intensity and morphological characteristics and, under some conditions, have elevated circulating androstenedione concentration when compared to residents. In this study we explore the role of androstenedione, an androgen precursor, and expression of satellite mating behaviors by experimentally manipulating androstenedione concentration in ruffs. Experimentally elevated androstenedione concentration, resulted in higher aggression and display frequency in the more aggressive resident phenotype, but did not elicit aggression in satellite male ruffs. Females implanted with androstenedione, expressed either resident or satellite behavior consistent with an autosomally inherited single locus dominant gene model, regardless of whether they were primed with testosterone. These results suggest androstenedione operates via conversion to testosterone at target tissues, and highlights the role of testosterone responsiveness of individual traits in the evolution of mating strategies in ruffs.

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Introduction

Androgens play an important role in expression of mating behaviors in male birds. Testosterone concentration is closely tied to aggression and mate acquisition during the breeding season (Van Duyse et al., 2002; Wingfield, 2005a). Within an individual, variation in breeding behavior may be a consequence of fluctuations in the concentration of testosterone (Deviche and Parris, 2006; Stoehr and Hill, 2000), testosterone receptors (Ball and Balthazart, 2004), enzymes (Fusani et al., 2003; Soma et al., 2003), binding molecules (Deviche et al., 2001), or the presence of second steroid precursors and metabolites that interfere with testosterone production or efficiency (Deviche et al., 2001). This relationship is further influenced by genetic effects on hormone sensitivity (Charlier et al., 2006) and affinity to produce testosterone (Jawor et al., 2006). The nexus between endocrine function, behavior and genetics makes the endocrine system an important mechanism underlying behavioral polymorphism.

A successful breeding season requires an energetic balance between reproduction and self maintenance. Testosterone facilitates a large number of traits associated with breeding in male birds (Alonso-Alvarez and Velando, 2001; Van Duyse et al., 2002), but prolonged exposure comes with both behavioral and physical costs (Wingfield et al., 2001). Under adverse social or environmental conditions, when costs associated with reproduction are too high, testosterone synthesis and breeding behaviors may be reduced (Wingfield, 2005b). Commonly, corticosterone is important in suppressing testosterone effects on behavior when conditions are not suitable for reproduction (Wingfield, 2005b), and provides a mechanism of behavioral polymorphism in some species (Knapp et al.,

2003). In ruff sandpipers, corticosterone is not associated with behavioral differences between strategies (Chapter 2), but androstenedione concentration does differ between behavioral phenotypes during the breeding season under some conditions (Chapter 1).

Ruffs are lekking shorebirds with three fixed genetically determined reproductive strategies (Lank et al. 1995, Lank et al 1999, and unpublished data). Resident males defend territories on a lek and perform silent displays in order to attract receptive females. Satellite males do not defend territories but form associations with resident males in order to gain access to females. A satellite males success depends on his ability to form an association with a resident male, his speed in mounting soliciting females and possibly female choice. A third phenotype, called "faeders", are small males that resemble females in appearance, do not defend territories and do not perform typical male breeding displays. Satellite males have elevated androstenedione concentration when housed in large flocks during the breeding season and do not show the same relationship between androgen concentration and social conditions seen in resident males (Chapters 1 & 2).

Lower androgen concentration in less aggressive ruff phenotypes is consistent with previous research on the role of testosterone, aggression and territory defense (Wingfield et al., 1990; Wingfield et al., 1982). However, previous studies have not defined a clear relationship between androstendione and breeding behavior in birds. Differences in androstenedione concentration among fixed breeding phenotypes raise the question of this hormone's role in the expression of alternative phenotypes. Androstenedione is a steroid that is a precursor for other steroid hormones and may be

converted to testosterone by the enzyme 17β-hydroxysteroid dehydrogenase (HSD) or to estradiol with aromatase (Matsunaga et al., 2002). If androstenedione is converted to testosterone in the brain, particularly in areas associated with aggression (Soma et al., 2003), it may stimulate behaviors typically associated with testosterone, while not leading to elevated levels of circulating testosterone. In contrast, if androstenedione is converted to estrogen or 5-alpha-dihidrotestosterone, it may activate entirely different behaviors, such as those characteristic of satellite male ruffs.

In this study we test the hypothesis that androstenedione activates satellite specific behaviors.. We use exogenous androstenedione to detect its relationship with behaviors typically associated with testosterone in male ruffs, and also to evaluate its ability to stimulate male breeding behaviors in female ruffs. Testosterone elicits both resident and satellite male breeding behaviors in female ruffs dependent on the female's pedigree (Lank et al., 1999). Androstenedione may also stimulate breeding behaviors in male ruffs via conversion to testosterone or another steroid hormone. If it is naturally present in high concentrations in satellite male ruffs and serves to stimulate satellite specific behaviors, then exogenous androstenedione would be expected to activate satellite specific behaviors in both males and females, regardless of genetic morph.

Methods

On 20 May, 2006, we implanted 4 captive male ruffs with androstenedione in order to observe the effects of androstenedione on reproductive behavior. Female ruffs underwent a parallel experiment, and were implanted with either testosterone or androstenedione on 27 June. All male and female ruffs were part of a long term captive

flock housed in an outdoor aviary at Simon Fraser University and previously exposed to quasi normal breeding conditions (Lank et al., 1999; Lozano and Lank, 2004). Males and females were housed in same sex pens for two months prior to the onset of the experiment.

Prior to implanting males with exogenous androstenedione, all captive males were observed for 20-40 minutes each day for three days. Seven males that were between 6 and 8 years of age, in full breeding plumage and had begun to express some breeding behaviors (full or partial displays) were selected to receive implants. Males were randomly assigned to each treatment group. On 20 May, we implanted two satellite and two resident males with 7 mm long silastic tubing (Dow Corning, 0.147 cm inner diameter, 0.197 cm outer diameter) packed with granulated androstenedione (Sigma) and sealed on both ends with silastic glue. We also implanted one satellite male and two resident males with empty silastic tubing of the same dimensions to serve as controls. All implants were inserted subcutaneously between the scapula and spine. A single 300 μl blood sample was collected from the brachial wing vein just prior to implanting.

All seven males were housed communally before and after implantation. We collected post-implant plasma samples two, five, eight and 11 days after implantation to monitor changes in androstenedione and testosterone. Implants were removed after 11 days.

Daily focal observations were conducted between 0700–1100 and 1500–1800, when two females were introduced into male pens. During each 10 minute focal observation we recorded all aggression, display and mounting behaviors (see Lank et al.,

1999 for a detailed description of behaviors). On 27 June, we implanted 24 female ruffs with either testosterone or androstenedione. Female ruffs were housed in all female flocks prior to implantation, and randomly selected from a pool of females that were between 2 and 8 years old and not currently laying eggs. We implanted six females with androstenedione and eighteen females with testosterone. Testosterone has previously been demonstrated to induce male typical breeding behaviors in female ruffs, consistent with autosomal inheritance of breeding strategy (Lank et al., 1999). Thus, females implanted with testosterone for the full two weeks (T treatment) served as controls to which we were able to compare the effects of androstenedione implanted females. A second group of six females retained testosterone implants for one week, followed by one week without any exogenous hormones (T/none treatment). These females remained part of the experiment after implants were removed, but served to simulated conditions in which an initial surge in testosterone is followed by a drop in testosterone, and reproductive behavior is potentially stimulated via other steroid hormones. A third treatment group, retained T implants for one week, and then switched to AE implants (T/AE treatment). These females mimicked the scenario in which an initial testosterone surge is required to elicit male reproductive behaviors, but subsequent stimulation can come from androstenedione. The fourth group of six females retained androstenedione implants for the entire study (AE treatment). Eighteen of the 24 implanted females were of previously known testosterone-induced behavioral male phenotype. Ten females of known phenotype were evenly distributed among T /AE and AE treatment groups. Remaining treatment groups each received a randomly selected group of four females of

known phenotype. With the exception of the six females whose T implant was removed after the first week all females were implanted for 14 days. Implants were of the same dimensions and materials as those used for males.

Prior to implanting, we collected a 300 µl blood sample from all 24 females. A second 300 µl sample was collected 7 days after implanting, and a final sample was collected on day 14 to assess whether implants were delivering either testosterone or androstenedione.

During the two weeks that females were implanted, three non-implanted females were housed with implanted females to serve as a behavioral stimulus. We conducted daily focal observations between 0600 and 1100 during which individual females were observed in the same manner as implanted males. After each 10 minute observation, a behavior score was calculated for each individual, with scores approaching 1 being indicative of a resident phenotype (Lank et al., 1999). At the end of the two week observation period we calculated an average behavior score, using only those observations conducted after a female began displaying male typical behaviors. A behavior score less than 0.8 was considered indicative of satellite behavior.

We collected all plasma samples using a 3 ml, syringe with 27 g detachable needle. Three hundred microliters of blood was collected from the brachial wing vein. Upon collection, samples were placed on ice until centrifuged and plasma was separated using a glass pipette. All plasma samples were frozen at -20° C until assayed at the University of California, Davis for androstenedione and testosterone. Hormones were extracted using dichloromethane, separated using column chromatography and assayed

by radioimmunoassay using testosterone and androstenedione specific antibodies (Sigma) (Goymann and Wingfield, 2004; Wingfield and Farner, 1975). Minimum detectability was 0.19 ng/ml for testosterone and androstenedione. Hormone recovery ranged from 30 – 81% for testosterone and 32-96% for androstenedione. Inter-assay coefficient of variation was 7% for testosterone and 19% for androstenedione. Intra-assay coefficient of variation was less than 5% for testosterone and androstenedione.

Repeated measures mixed effects models (SAS ProcMixed) were used to examine the relationship between hormone concentration and date for males implanted with AE. Individual was included as a random factor. A chi-square test was used to evaluate differences in behavior frequencies among treatment groups, and linear regression was used to analyze the relationship between AE and androgen concentrations as well as previous and current female behavior scores.

Results

Male Physiology

Prior to implantation, average male testosterone concentrations were 0.16 ± 0.05 ng/ml for satellite males and 0.26 ± 0.23 ng/ml for resident males. Average androstenedione concentration for satellite males was 1.1 ± 0.96 ng/ml and 0.49 ± 0.17 ng/ml for resident males.

For resident males, androstenedione was elevated in AE-implanted ($F_{1,2} = 15.94$, $P = 0.05$ Figure 3.1) compared to control individuals. However, androstenedione concentration did not differ between control and implanted satellite males ($F_{1,1} = 0.37$, P

= 0.65; Figure 3.1). Androgen concentration did not change over time for satellite males ($F_{4,3} = 0.32$, $P = 0.84$), but was elevated post implant in both treated and non-treated resident males ($F_{4,8} = 5.51$, $P = 0.02$). Testosterone was higher in resident males than in satellite males after implantation ($F_{1,5} = 44.75$, $P < 0.01$; Figure 3.1), but was not significantly related to AE concentration for any treatment group (Figure 3.2). Androgen concentration did not differ between control and implanted individuals for either residents ($F_{1,2} = 0.51$, $P = 0.55$) or satellites ($F_{1,1} = 0.37$, $P = 0.65$).

Behavior

Both satellite and resident male ruffs implanted with AE had higher display frequency post-implant than did non-implanted males ($X^2 = 18.88$, $df = 10$ $P = 0.04$ for resident males, $X^2 = 37.12$, $df = 10$, $P < 0.01$ for satellite males). Implanted resident males also had higher aggression frequencies than did non-implanted resident males ($X^2 = 18.90$, $df = 10$, $P = 0.04$). Neither implanted nor control satellites exhibited any aggressive behaviors during this study.

Sixteen of the 24 females implanted with AE or T exhibited male behaviors within three days of being implanted. Implantation with AE or T produced both resident and satellite behavior in females regardless of implant type (Figure 3.3). Behavior scores ranged from 0.03 to 1. Nine females of known phenotype responded to implants with male behavior. All nine of these females were assigned to satellite and resident

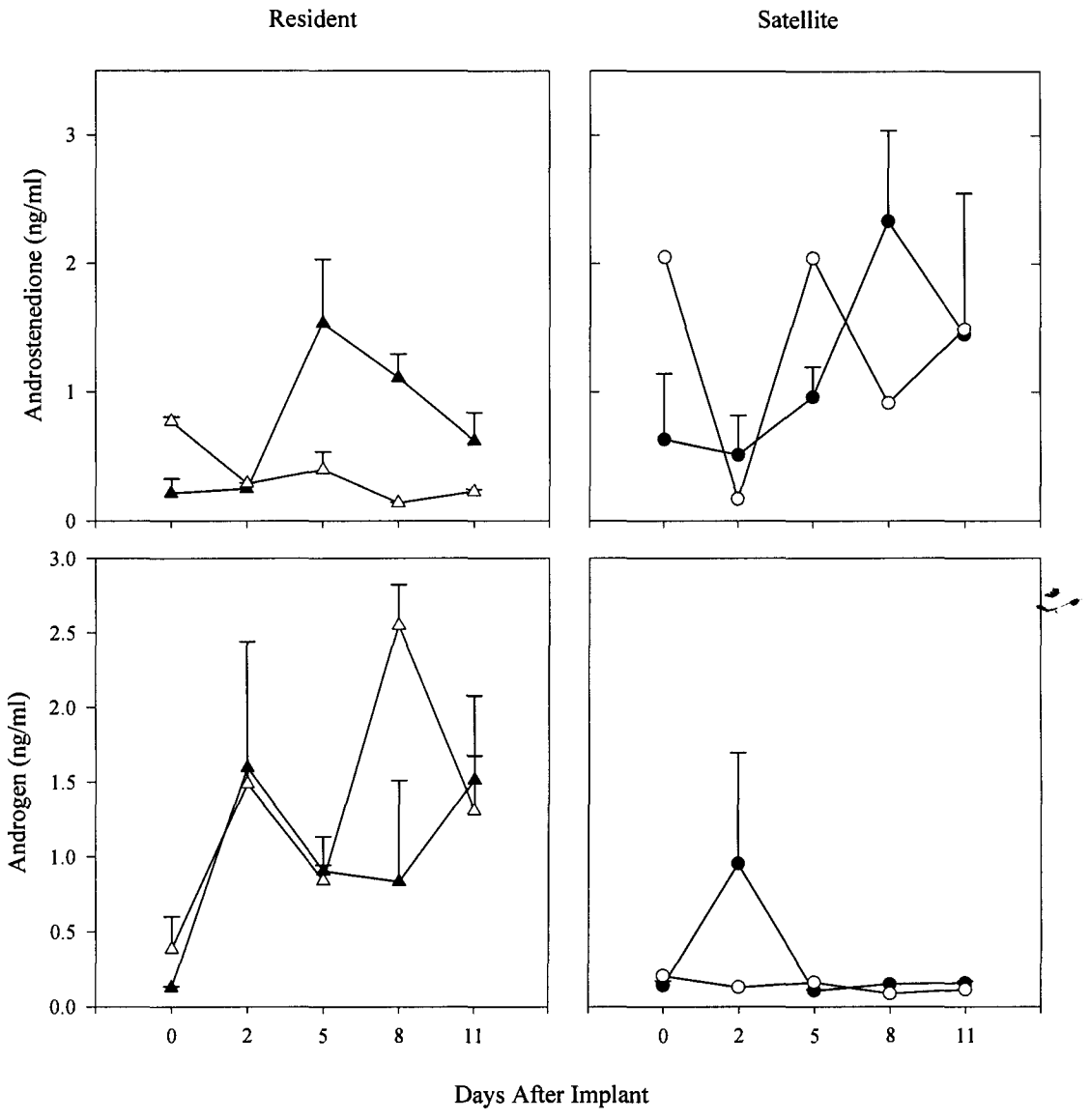

Figure 3.1 Mean androstenedione and androgen concentration for androstenedione implanted male ruffs. Implanted resident (filled triangles) and satellite (filled circles) as well and non-implanted resident (open triangles) and satellite (open circles) male ruffs are shown. Error bars indicate standard error.

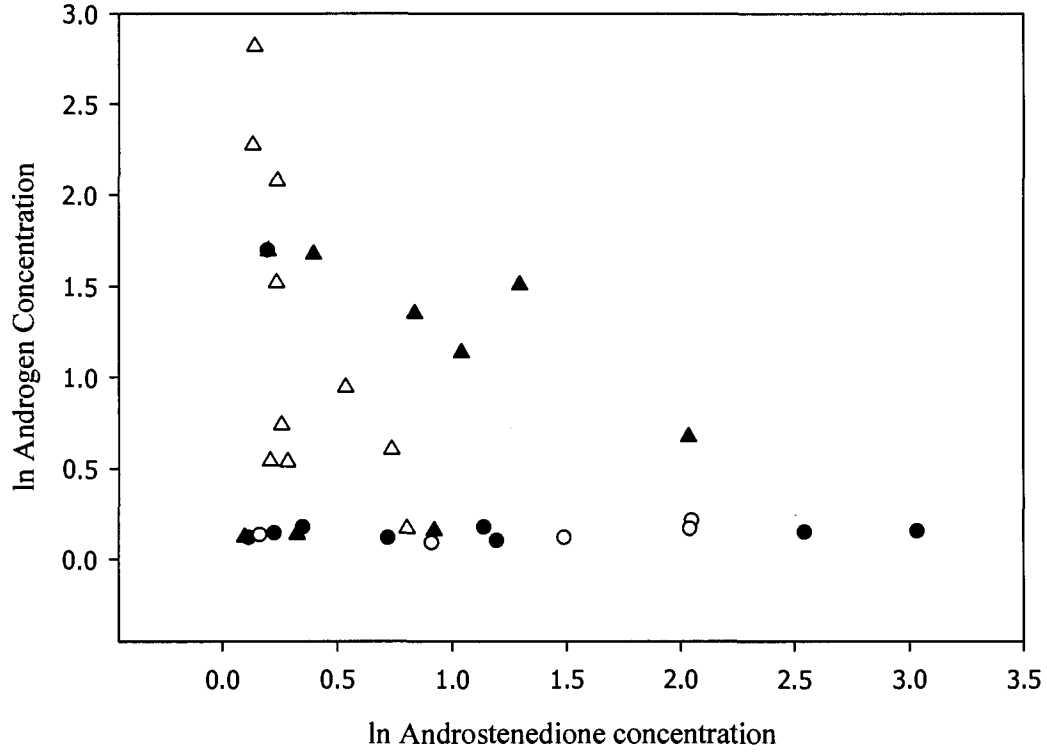

Figure 3.2 Androstenedione vs. androgen concentrations for male ruffs. Residents are represented with triangles. Satellites are represented with circles. Filled symbols correspond to androstenedione implanted individuals.

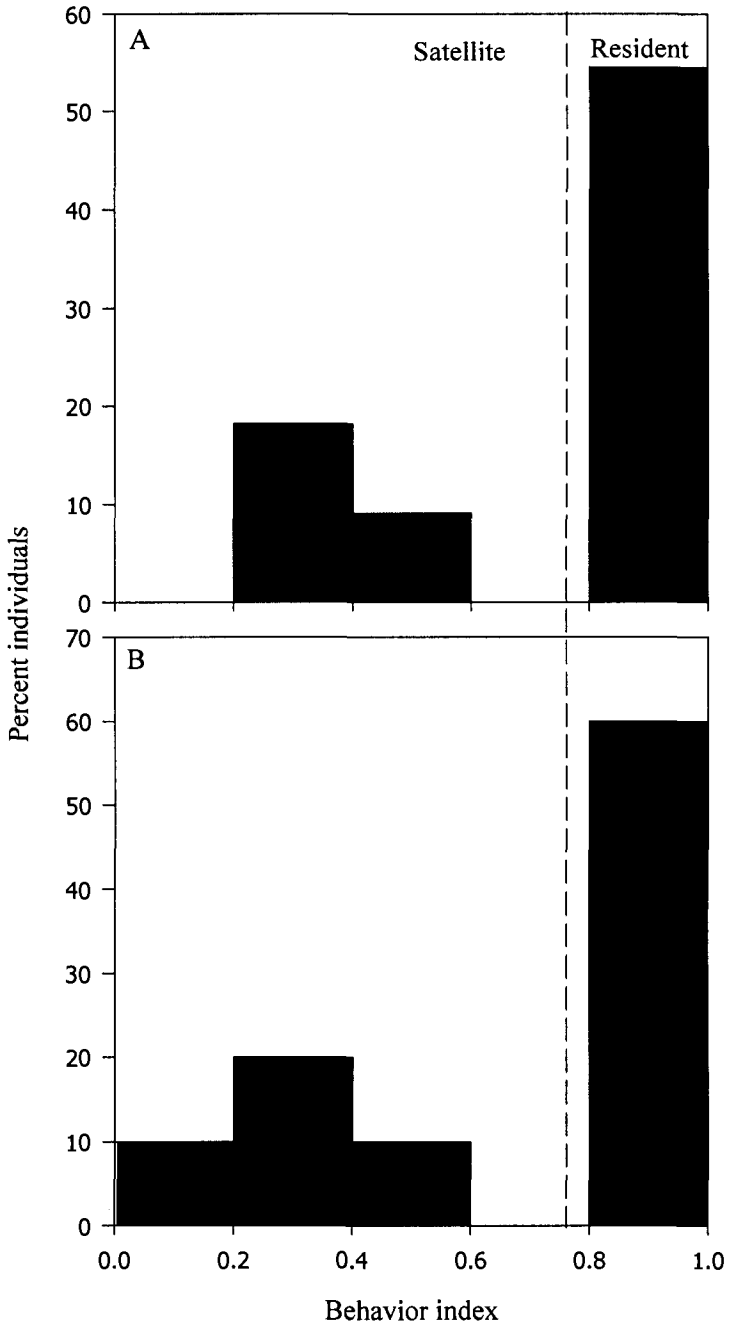

Figure 3.3 Distribution of implanted female behavior scores. Females were implanted with testosterone (A, n = 6) or androstenedione (B, n = 11) for 14 days. Behavior scores are based on Lank et al. (1999). Behavior scores equal to or below 0.8 indicated satellite phenotype. Those above 0.8 indicate resident phenotype.

phenotypes consistent with previous assignments. In addition, previous (Lank et al. 1999 and unpublished) and current behavior scores were significantly correlated ($r = 0.77$, $P = 0.02$, Figure 3.4)

Display and aggression frequencies did not differ between treatment groups ($X^2 = 23.40$, $df = 30$, $P = 0.80$ for display behavior; $X^2 = 24.8$, $df = 30$, $P = 0.73$ for aggression). Display frequency dropped to zero three days after T implant removal for all individuals, but aggressive behaviors remained in some individuals for four days after implant removal. Females initially implanted with testosterone, then switched to androstenedione, showed no decline in display or aggression frequency and had behavior indices similar to females with continuous testosterone or continuous AE implants. Non-implanted females did not exhibit any male typical breeding behaviors, but did solicit copulations from both T and AE implanted females.

Discussion

In male ruffs, exogenous androstenedione did not alter the expression of genetically determined phenotypes, nor did it alter circulating testosterone concentration by resident males. Males given exogenous androstenedione had higher display or aggression frequencies than did males not receiving an implant. Both androstenedione and testosterone stimulated resident and satellite male breeding behaviors in female ruffs consistent with known and predicted phenotypes. These results suggest androstenedione operates as a precursor, altering testosterone concentrations at target tissues without

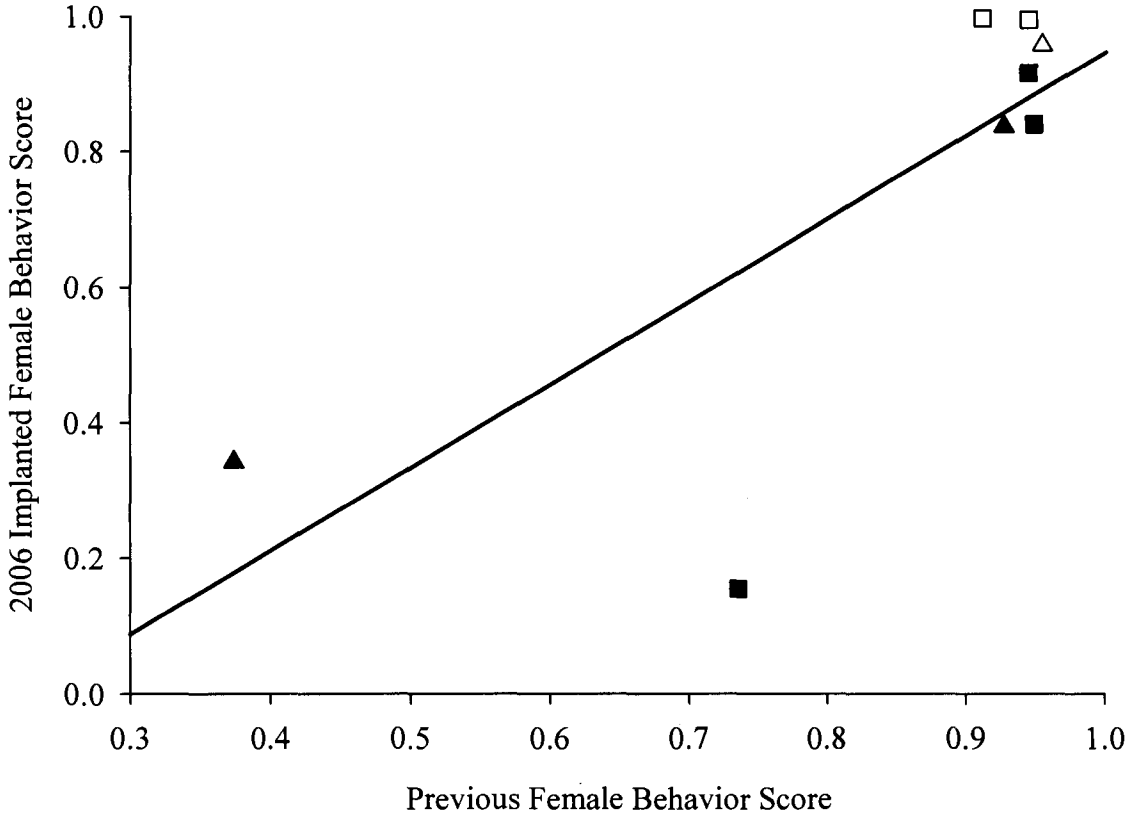

Figure 3.4 Previously determined female behavior score (Lank et al. 1999) vs. female behavior scores determined in 2006. Filled triangles are T/T treated females, empty triangles are T/C treated females, filled squares are T/AE treated females, and empty squares are AE/AE treated females

altering plasma androgen concentrations. Additionally, these data are consistent with the idea that conversion of hormone precursors to active hormones at target tissues may be a major mechanism to avoid high levels of sex steroids in the blood for prolonged periods.

In previously studied systems, testosterone plays a significant role in facilitating breeding behavior in male birds (Pinxten et al., 2003; Wingfield, 2005a), particularly when aggression is an important part of mate acquisition. However, for some species, testosterone does not increase above baseline levels during the breeding season (Moore et al., 2004a; Wingfield et al., 1982), or the relationship between circulating testosterone concentration and some breeding behaviors are dissociated (Moore et al., 2004b; Wiley and Goldizen, 2003). The absence of elevated circulating testosterone concentrations during the breeding season raises the question of what physiological factors underlie the expression of key mating behaviors in the absence of testosterone, and what evolutionary forces are driving these differences in association between steroid hormones and behavior.

When suites of behaviors and physical characters are mediated by the same hormones, it can be difficult to distinguish what traits were under selection (Ketterson and Nolan, 1999). Evolutionary changes in circulating hormone concentration may influence a large number of behaviors, permitting traits not under direct selection to be carried along by correlated traits (Ketterson and Nolan, 1999). In contrast, the evolutionary potential hypothesis suggests dissociation between circulating hormone concentration and trait responsiveness may permit individual characters to evolve independently (Hau, 2007). Changes in receptor density or enzyme activity at target

tissues allow selection to occur on isolated traits. In ruffs, circulating hormone concentrations differ between phenotypes, but changes occurring at target tissues may lead to further differentiation in reproductive behavior and physiology.

Lank et al. (unpublished data) previously found that implanting males with T, accelerated the seasonal development of breeding plumage and behavior, but did not alter individual resident or satellite phenotypes. In this study administering exogenous testosterone or androstenedione to female ruffs did not alter previously established suites of characters associated with alternative phenotypes. Individual, phenotype-specific traits are expressed in a manner consistent with previously established behavioral phenotypes or predicted phenotype based on parentage. In resident male ruffs, testosterone (Chapter 2) and androstenedione stimulate aggression and territory defense, while in satellite male ruffs these same hormones fail to stimulate either behavior. These results suggest that the behavioral differences between resident and satellite ruffs are not a consequence of differences in circulating hormone concentrations, but rather alterations in the capacity to respond to each hormone at select target tissues.

Elevated circulating testosterone concentration comes at a physiological and ecological cost (Deviche and Parris, 2006; Wingfield et al., 2001). As part of the biosynthetic pathway in the synthesis of testosterone and estrogen, androstenedione may be released into the bloodstream prior to conversion to either testosterone or estrogen, providing the substrate for conversion at target tissues, while avoiding elevated T or estrogen concentration in the bloodstream. Conversion of androstenedione to an active testosterone requires the presence of the enzyme 17β-hydroxysteroid dehydrogenase

(HSD) (Matsunaga et al., 2002). HSD isozymes are present and operate in normal adult gonads, as well as other tissues. The stimulation of testosterone associated behaviors such as display frequency by androstenedione in satellite ruffs suggests that HSD isozymes are present in target tissues associated with some mating behavior, but are perhaps lacking or in low quantities in other areas of the brain. Low circulating levels of androgens in satellite males further suggests HSD isozymes may be low in the gonads. Conversion in the gonads leads to elevated circulating testosterone levels, while conversion at target tissues would stimulate behaviors specifically beneficial for reproduction, while reducing physiological and ecological costs associated with prolonged elevated T concentration. Considering HSD activity and testosterone receptor density in various tissues across phenotypes may be a promising avenue for identifying traits under selection in the evolution of alternative reproductive strategies in the ruff.

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General Conclusion

This study provides two novel contributions to the study of evolutionary and behavioral physiology. First it highlighted the role of androstenedione in facilitating the expression of alternative reproductive strategies, which has not previously been demonstrated. Second, results from experimental manipulations suggest expression of alternative reproductive strategies in the ruff is a result of the capacity of various brain regions to respond to steroid hormones or convert inactive precursors into active hormones. These results, furthermore, are consistent with the idea that dissociation between circulating androgen concentration and trait responsiveness may operate as a mechanism facilitating behavioral variation when elevated androgen exposure would be costly. These results highlight the need to examine the role of androstenedione in mating behavior, particularly in species or individuals with little or no territory or mate defense.

Seasonal hormone profiles for resident male ruffs did not mimic those found in males of other polygamous (Johnson, 1998) breeding systems, but did show an association with conditions within the lek. An increase in androgen concentration may facilitate territorial behaviors, but shows considerable fluctuation in timing between individuals. Timing of peak breeding effort may be influenced by previous breeding activities (Vitousek et al., 2008) as well as present social conditions (Boseret et al., 2006; Caro et al., 2007). The intensity of behavior and energetic demands found on leks may further skew the timing of breeding in individuals.

Androstenedione, proved to be an influential hormone in this mating system; however it has received little study in adult birds (but see Goymann and Wingfield, 2004). As a precursor to both estrogen and testosterone, with no known specific receptor of it's own, the effects of androstenedione depend largely on the enzymes and receptors present at target tissues. A loss of 17β-hydroxysteroid dehydrogenase enzymes in the gonads may affect production of testosterone and subsequently a large number of characteristics typically stimulated by testosterone, but may also reduce prolonged exposure to androgens when high concentrations might be ecologically or physiologically costly. In ruffs as well as other birds, aggression is linked to testosterone in temperate breeding systems, while mating displays and copulation frequency show less of an association (Moore et al., 2004; Wiley and Goldizen, 2003). Differences in concentration or type of enzymes present in brain regions promoting mating postures and copulation behavior may permit some behaviors to be expressed in the absence of elevated circulating testosterone concentration when precursor molecules are present in sufficient quantity to be converted at target tissues.

Hormonal mechanisms underlying alternative reproductive strategies appear to be less consistent across taxa than the patterns observed across mating systems. In lizards, sensitivity to corticosterone is at least partially responsible for behavioral differences between mating phenotypes (Denardo and Sinervo, 1994a; Denardo and Sinervo, 1994b); however, this relationship was not detected in ruffs. More research will be required before we may detect similarities in endocrinological pathways associated with alternative mating strategies across taxa. This research is likely a promising pathway as

we begin to document the degree of behavioral variation found within mating systems and species. Endocrinological variation found in association with alternative mating tactics likewise may provide an index of the plasticity of mating behavior and degree of variation available for selection.

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